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Original Article

A STUDY ON ELECTROPHORESIS ANALYSIS OF ACID PHOSPHATASE ISOZYMES DURING DIFFERENT DEVELOPMENTAL STAGES OF KALIMPONG-A (KA), NEW BIVOLTINE-18 (NB_{18}), AND PURE MYSORE (PM) LINES OF *BOMBYX MORI* L

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ABSTRACT

The molecular data, in particular gel electrophoresis of enzymes and numerical methods of analysis, have proven useful in many groups of insects and will see much wider use in future. Therefore, the present study was designed with the main purpose to analyze the activities of acid phosphatase isozymes by electrophoresis method during different developmental stages of Kalimpong-A (KA), B_{18}, and Pure Mysore (PM) of Bombyx mori L. Standardized disc electrophoresis method was performed. Acid phosphatase isozymes form distinct enzymes zones in the photographs and in the zymogram and these have been numbered in cathodal to anodal sequence. These isozyme patterns have been established after repeated runs. The total isozymes of different developmental stages of KA, NB_{18}, and PM have been grouped into different zones. The nomenclature of enzyme banding pattern has been followed. Results delineated that changes in ACPH zymograms during development stages revealed a total of 19 bands in KA & PM, 18 bands in NB_{18}. Furthermore, it was evident from the results of the present study that ACPH activity was low in KA. ACPH activity was moderate in larval as well pupal stages. Moreover, in the pupal stage, the ACPH activity was less in male pupae of all the races and it was found to be high in female pupae of all the races.

KEYWORDS: Bombyx Mori L, Acid Phosphatase, Electrophoresis, Kalimpong-A (KA), New Bivoltine-18 (NB_{18}) & Pure Mysore (PM)

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INTRODUCTION

A study on the taxonomy of closely related species is important though difficult. Earlier studies on the entomological taxonomy were based on morphologically defined species, the degree of morphological difference being the essential criterion. This topological approach still dominates date to day practices of most systematics. However, this concept has failed to deal adequately with sibling or cryptic species.[1] In such cases the use of biochemical study has been considered of some use in the separation of closely related species of insects.

Reliable methods for distinguishing members of the insect complex by chromatographic studies of pteridine species have been attempted which help to identify the members of the complex.[1-4] Immuno diffusing techniques have been used to separate insect species in particular mosquitoes.[5,6] Schumann (1973) analysed through gel diffusion techniques different strains of mosquitoes of different origins and identified them.[7]

A science concerned with establishing durable classification has itself undergone tremendous changes in the last three decades. The traditional approach still is the basis of all taxonomical studies. The molecular data, in particular gel electrophoresis of enzymes,[8-13]and numerical methods of analysis have proven useful in many groups of insects and will see much wider use in future.[14] With this background, the current study was planned with the main purpose to analyze the activities of acid phosphatase isozymes by electrophoresis method during different developmental stages of new breeding lines and races of *Bombyx mori* L.

MATERIALS AND METHODS

Silkworm Varieties and Rearing

The pure races of bivoltine Kalimpong-A (KA) spinning oval white cocoons, New Bivoltine-18 (NB_{18}) spinning dumbbell white cocoons and multivoltine Pure Mysore (PM) spinning pointed yellow cocoons of mulberry silkworm Bombyx *mori* L. were selected for the present breeding programme. These races were obtained from their respective seed areas and are reared in cytogenetics laboratory, Jnana Bharathi, Bangalore University. The disease free layings were prepared as described by Krishnaswamy, and were incubated at 25°C and relative humidity of 60-70%. On the 8th day, composite layings were prepared (10-20 layings were prepared 100-200 eggs were collected from each laying). The hatched worms were reared according to the method described by Krishnaswamy. [15]MS varieties of mulberry leaves were used in rearing. The worms were reared in mass up to III instar, after III moult 300 worms were collected in three replicates in order to evaluate the rearing performance. Standard temperature and humidity were maintained in the rearing house.

Preparation of Enzyme Extract

The different developmental stages such as 1st day, 5th day and 9th day eggs, five larval instars (I, II, III, IV, and V instars), early, middle and late stages of male and female pupae, male moths before and after copulation. Female moths before and after egg laying of bivoltine races KA, NB_{18}, and multivoltine race PM were selected.

Electrophoresis

Disc electrophoresis was performed essentially according to Davis (1964) and Ornstein (1964).[16,17]A discontinuous gel system consisting of 7.5% lower gel and 3.12 5% spacer gel was used. The lower gel consisted of one part of Tris-hydrochloric acid buffer (36.g Tris+ 48.0 ml of N HCl + 0.46 ml of TEMED, diluted to 100ml. pH 8.9), two parts of cyanogum 41 (3.08 g of cyanogum in 10ml of water), two parts of Ammonium persulphate (140mg of APS in 100ml of water) and three parts of distilled water. 1.2 ml of this solution was poured into clean, dry glass tubes (7 cm x 0.7 cm dia) held vertically. The solution was carefully over layered with distilled water and allowed to photopolymerise for 15 minutes under a fluorescent lamp or daylight. After polymerisation, the water layer was removed from the top and the spacer gel was added. The spacer gel consisted of 1 part of Tris phophoretic acid buffer (5.7 g tris + 25.6 ml of 1M H3Po4 + 0.46 ml of TEMED diluted to 100 ml with distilled water pH 6.9) 2 parts of cyanogen 41 (1.25 g cyanogum 41 in 10ml of Distilled water) 1 part of APS (70 mg in 100 ml) and four parts of water. 0.2 ml of spacer gel was poured on the top of the lower gel each tube layered with a drop of water and allowed to Photopolymerise for 15 minutes. After polymerization, the water was blotted off and the tubes with spacer gel were inserted into the rubber connectors of the upper electrode vessel. The electrode chambers were filled with electrode buffer (0.3 M boric acid and sodium hydroxide buffer pH 8.65). The sample, suitably diluted with 20% sucrose containing bromophenol blue, was carefully layered onto each gel and subjected to electrophoresis in cold (4°C) imposing a current of 2mA per tube for 2 hours.

Staining Procedure

The stain of acid phosphatases (APH) constituted sodium 1-naphthyl phosphate 100 mg, polyvnylpyrrolidone 500 mg. Fast blue RR salt 100 mg. Manganese chloride 60 mg. Magnesium chloride 60 mg and sodium chloride 2 gms dissolved in 100 ml of 0.125 M acetate buffer at pH 5.0. The gels were incubated in the stain for 10-20 minutes until the bands appeared. The gels were then stored in 6% acetic acid. All pertinent gels were photographed and diagrammatic representation of the gels was presented in the form of zymograms as deeply stained, moderately stained and faintly stained bands.

RESULTS

The zymograms of the acid phosphatase (ACPH) of the race Kalimpong-A (KA) revealed 8 ACPH zones. ACPH 1 zone consists of 1 band. It is moderately stained in 288h female pupae. Band 3 is darkly stained in 24h, 144h, 288h female pupae. Band 3 is darkly stained in 24h, 144h, 288h female pupae. ACPH-2 zone consists of 3 bands (4,5 and 6). Band 4 is faintly stained in 24h male pupae, darkly stained in 216h, eggs, 288h, male pupae. Band 5 moderately stained in I instar larvae darkly stained in 288h male pupae. Band 6 is darkly stained in 288h male pupae. ACPH-4 zone consists of 3 bands (7,8 and 9). Band 7 1s absent. Band 8 is moderately stained in I instar larvae. Band 9 is moderately stained in II instar larvae, darkly stained in 24 male pupae. ACPH-5 zone consists of 3 bands (10,11 and 12). Band 10 1s darkly stained in II instar larvae. Band 11 1s darkly stained 1n 24h, eggs, III and IV instar larvae, 24h, 144h male pupae, 144h female pupae and male adult after copulation. Band 12 1s darkly stained in 24h, 216h eggs, I, III, IV and V instar larvae, 24h, 144h male pupae, 144h female pupae, male adult before copulation. ACPH-6 zone consists of 3 bands (13, 14 and 15). Band 3 is moderately stained in 120h eggs, IV, V instar larvae, 288h male pupae, darkly stained 1n II, III instar larvae, male adult before copulation. Band 14 is darkly stained 1n 216h eggs, II, IV instar larvae. Band 15 1s moderately stained in V instar larvae, and is darkly stained in III instar larvae. ACPH-7 zone consists of 3 bands (16, 17 and 18). Band 16 is moderately stained in 120h eggs, IV, V instar larvae, 288h male pupae, darkly stained in II, III instar larvae, female adult before oviposition. Band 17 1s moderately stained in IV instar larvae, darkly stained in 216h eggs. Band 18 is faintly stained in V instar larvae, male adult after copulation, darkly stained 1n 120h eggs, III instar larvae. ACPH-8 zone consists of 3 bands (19, 20 and 21). Band 19 1s faintly stained in female pupae of 24h, moderately stained 1n female adult before oviposition. Band 20 1s darkly stained 1n IV instar larvae. Band 21 1s are commonly present in all the developmental stages (Figures. 1, 2, and 3).

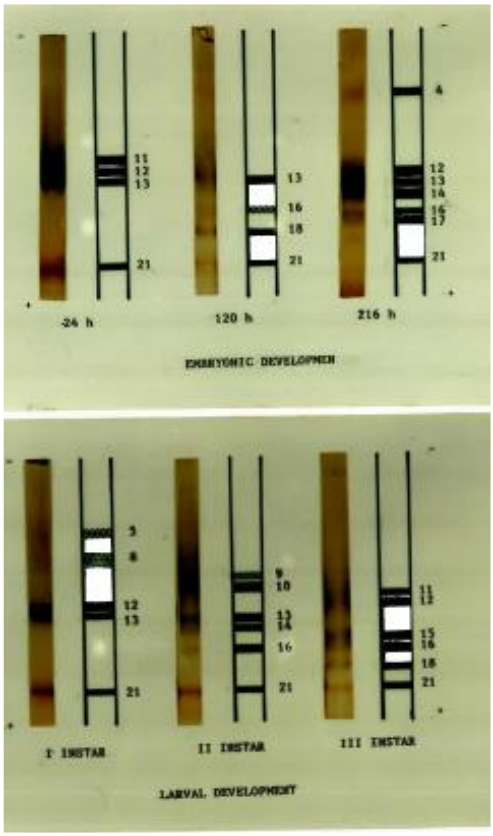

Figure 1: Acid Phosphatase Zymograms of Kalimpong-A (KA).

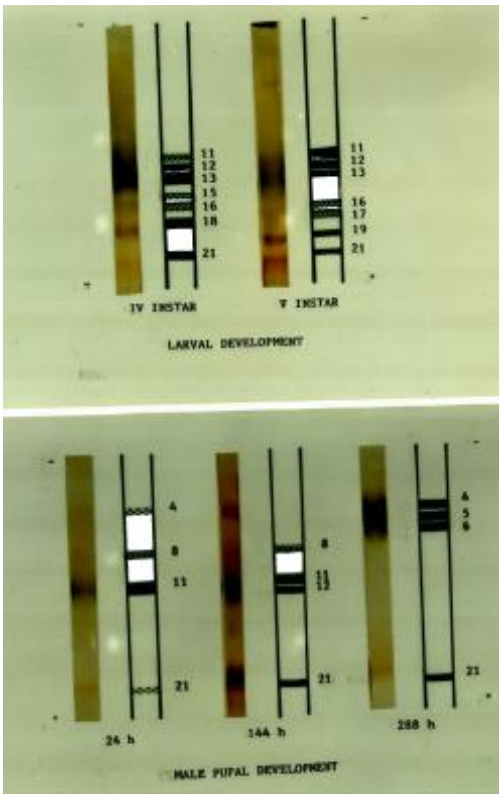

Figure 2: Acid Phosphatase Zymograms of Kalimpong-A (KA).

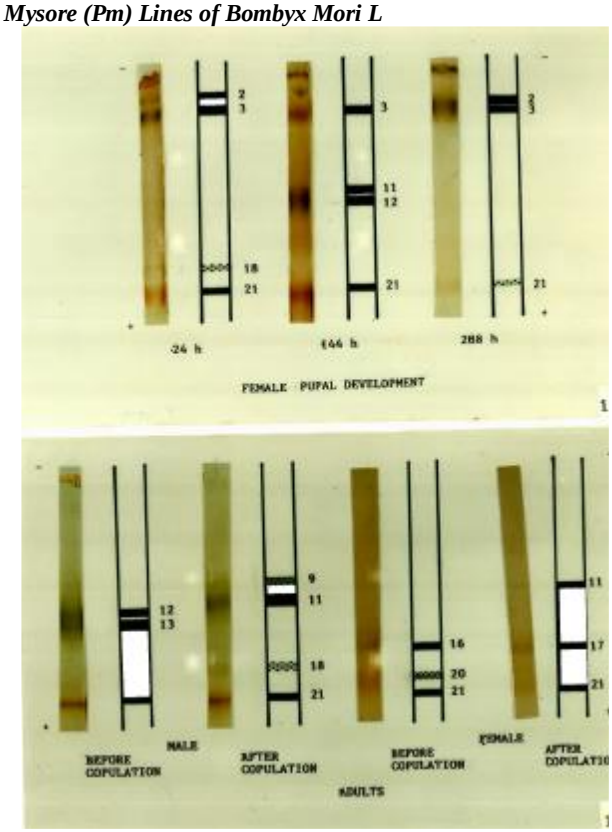

Figure 3: Acid Phosphatase Zymograms of Kalimpong-A (KA).

The zymograms of acid phosphatase isozymes of the race New Bivoltine-18 (NB_{18}) are grouped into 8 zones. ACPH-1 zone consists of no bands. ACPH-2 zone consists of 2 bands (2 and 3). Band 2 1s faintly stained in female pupae 144h, 288h, male adult after copulation. Band 3 is moderately stained in 120h eggs. ACPH-3 zone consists of 3 bands (4,5 and 6). Band 4 1s darkly stained in the male adult after copulation. Band 5 absent. Band 6 is moderately stained in male adults after copulation and female adult before and after copulation, darkly stained 1n 120h, 216h eggs 288h ale pupae. ACPH-4 zone consists of 3 bands (7, 8 and 9). Band 7 is doubly stained 1n 144h, female pupae, male adult after copulation. Band 8 is moderately stained in 216h eggs, male adult after copulation, doubly stained in I, V instar larvae. Band 9 is moderately stained in 288h male pupae, doubly stained in III, V instar larvae, 24h, 288h female pupae, male adult before copulation. ACPH-5 zone consists of 3 bands (10, 11 and 12). Band 10 is moderately stained in 144h male pupae, male adult after copulation, darkly stained in 216h eggs, II and III instar larvae, 24h female pupae. Band 11 is moderately stained in 24h eggs, 144h male pupae, darkly stained in II, V instar larvae, 24, 144h, male pupae. Band 12 is darkly stained in I, IV instar larvae, 24h male pupae and male adult after copulation. ACPH-6 zone consists of 3 bands (13, 14 and 15). Band 13 is faintly stained in 24h eggs, moderately stained in III instar larvae, darkly stained in 120h eggs, I, IV instar larvae, 144h, 288h female pupae. Band 14 is faintly stained in male adults after copulation darkly stained in 216h eggs, II instar larvae, female pupae of 288h. Band 15 is moderately stained in 24h, 120h eggs, II instar larvae, darkly stained in IV instar larvae. ACPH-7 zone consists of 3 bands (16, 17 and 18). Band 16 is moderately stained in 24h, 144h male pupae and 24h female pupae. Band 17 is darkly stained in 144h male pupae and 24h female pupae. Band 18 1s darkly stained 1n II instar larvae. ACPH-8 zone consists of 3 bands (19, 20 and 21). Band 19 is absent. Band 20 is darkly stained in 288h male pupae. Band 21 is common to all the developmental stages (Figures 4, 5, and 6).

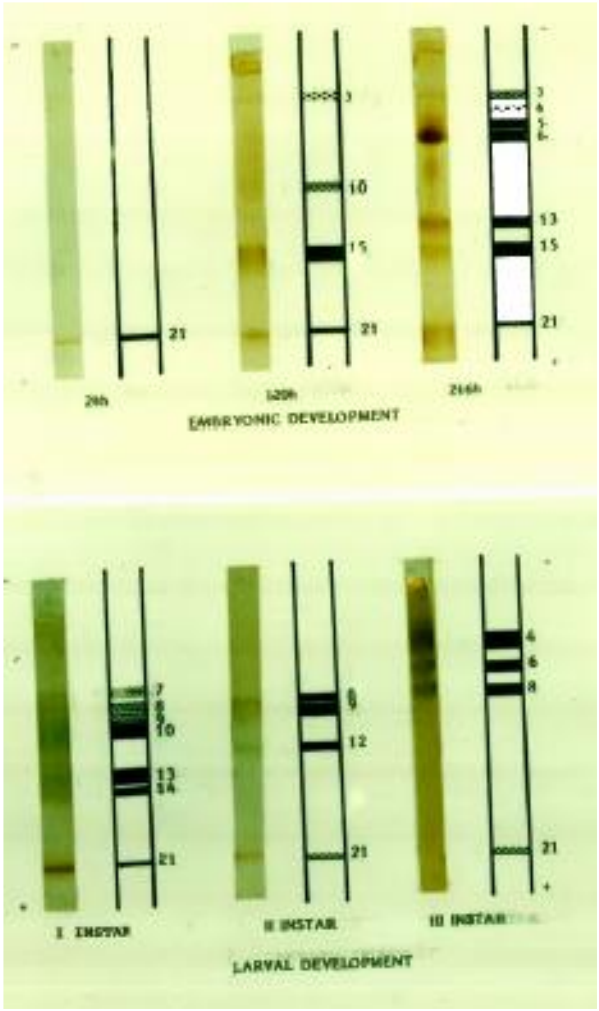

Figure 4: Acid Phosphatase Zymograms of NB_{18}.

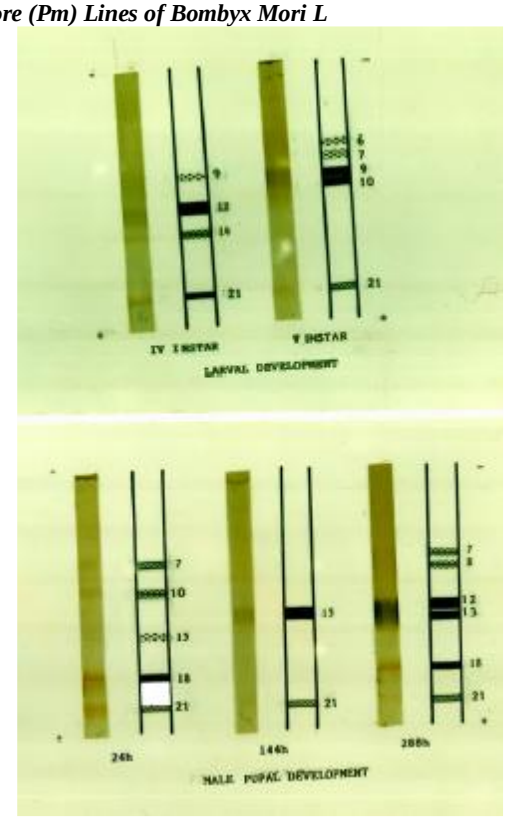

Figure 5: Acid Phosphatase Zymograms of NB_{18}.

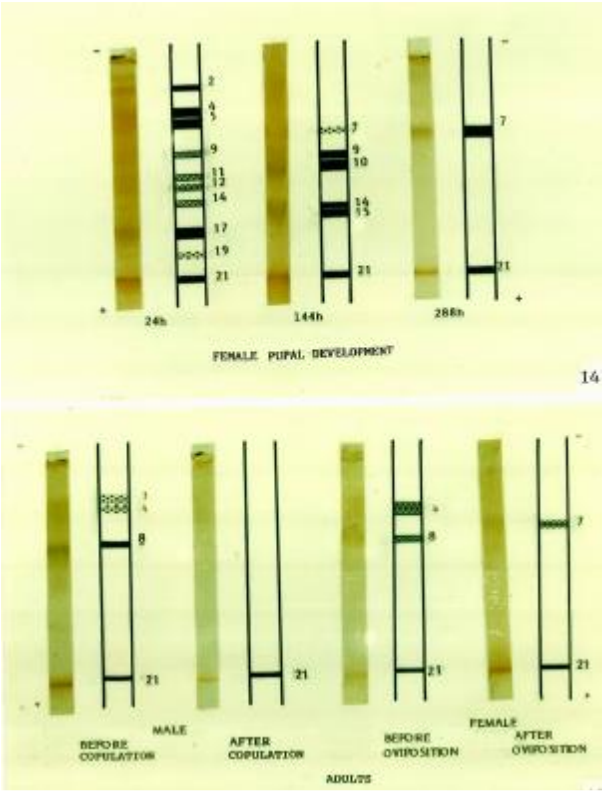

Figure 6: Acid Phosphatase Zymograms of NB_{18}.

The zymograms of the acid phosphatase of race pure mysore (PM) isozymes were grouped into 8 zones.ACPH-1 zone consists of one band (1) and is moderately stained in 24h, 144h male pupae. ACPH-2 zone consists of 2 bands (2 and 3). Band 2 1s moderately stained in 216h eggs, 288h male pupae, 24h female pupae. Band 3 is moderately stained in 216h eggs, II instar larvae, darkly stained in 24h male pupae. ACPH-3 zone consists of 3 bands (4,5 and 6). Band 4 1s faintly stained 1n instar larvae, darkly stained 1n 24h female pupae. Band 5 is darkly stained in 120h eggs, II instar larvae, 144h male pupae, 24h female pupae. Band 6 1s darkly stained in II instar larvae, 24h female pupae. ACPH-4 zone consists of 3 bands (7, 8 and 9). Band 7 is moderately stained 1n 120h, eggs, male adult before copulation and darkly stained in 288h female pupae. Band 8 is faintly stained in 144h female pupae, darkly stained in 288h female pupae. Band 9 is moderately stained in male adults before copulation and darkly stained in I instar larvae. ACPH-5 zone consists of 3 bands (10, 11 and 12). Band 10 is darkly stained in I, V instar larvae, 288h male pupae, male adult before copulation and female adult before oviposition. Band 11 is moderately stained in 120h eggs, 24h female pupae, darkly stained in 216h eggs, IV instar larvae, 24h male pupae and female adult, before oviposition. Band 12 is faintly stained in II instar larvae, doubly stained in III, IV, V instar larvae, male adult before and after copulation, female adult after oviposition. ACPH-6 zone consists of 3 bands (13, 14 and 15). Band 13 is moderately stained in 24h eggs, darkly stained in 120h, 216h eggs, III, V instar larvae, 144h female pupae, male adult after copulation, female adult after oviposition. Band 14 is moderately stained in 216h eggs, IV instar larvae, darkly stained in V instar larvae, 144h female pupae. Band 15 is absent. ACPH-7 zone consists of 3 bands (16, 17 and 18). Band, 16 is darkly stained in 216h eggs, IV instar larvae. Band 17 is darkly stained in 216h eggs. Band 18 is moderately stained 1n III instar larvae, darkly stained 1n 288h male pupae. ACPH-8 zone consists of 3 bands (19, 20 and 21). Band 19 is darkly stained in V instar larvae, 144h female pupae. Band 20 is absent. Band 21 1s commonly present 1n all the developmental stages (Figures 7, 8, and 9).

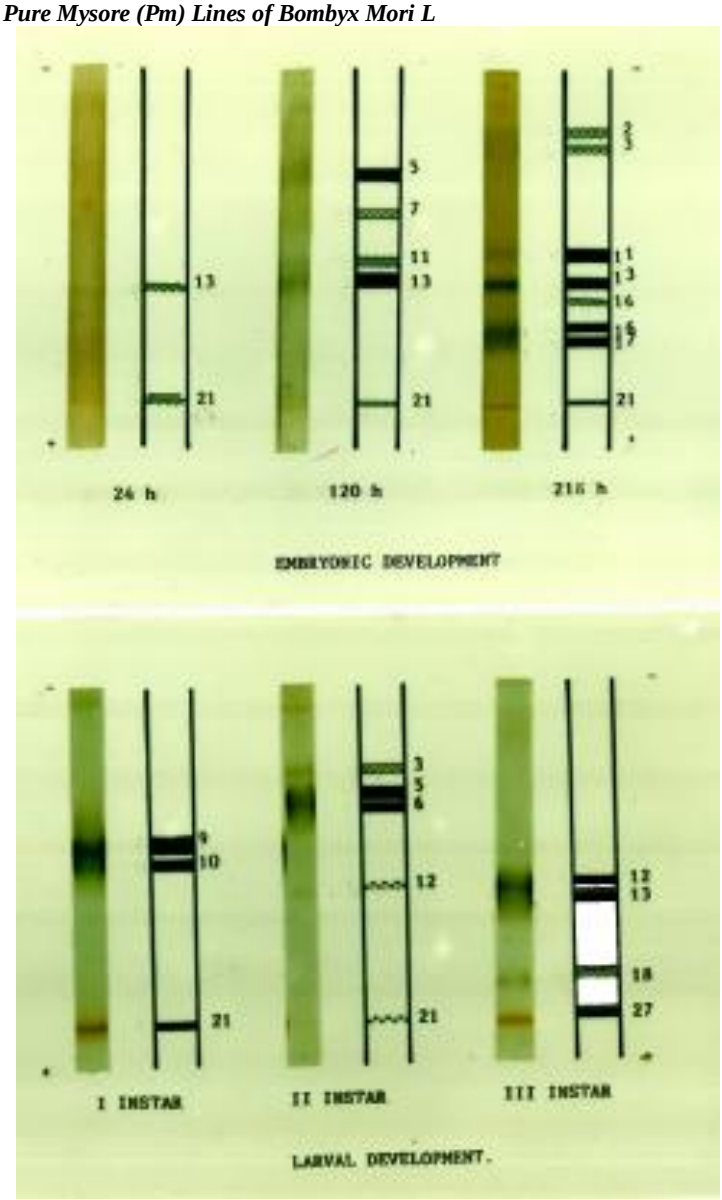

Figure 7: Acid Phosphatase Zymograms of PM.

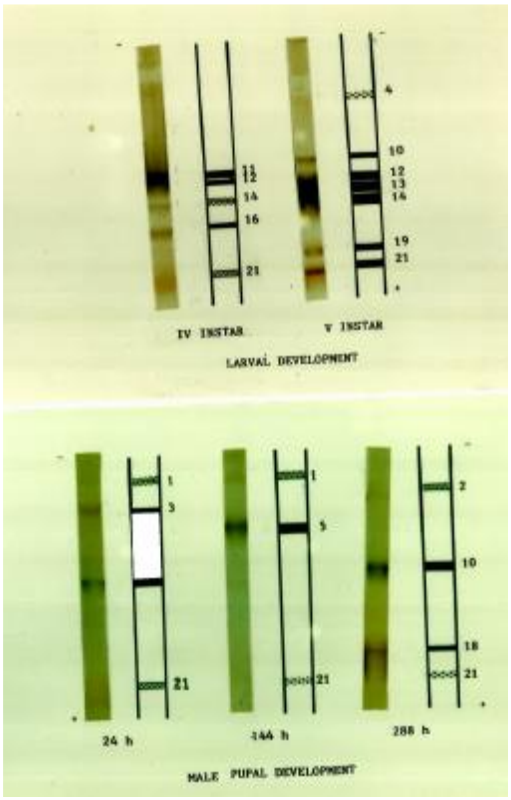

Figure 8: Acid Phosphatase Zymograms of PM.

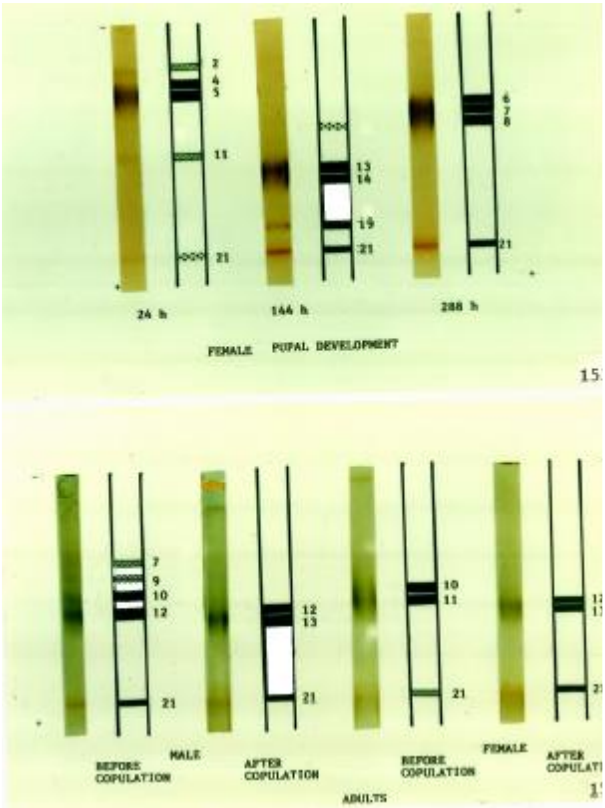

Figure 9: Acid Phosphatase Zymograms of PM.

DISCUSSIONS

The occurrence of phosphatases in silkworm Bombyx mori was first reported by Nakamura (1940)[18] later many workers studied these enzymes.[19-21] The results obtained showed increased activity from eggs to V instar larvae and it was decreased in pupae and adults. This is supported by other workers also.[22,23] The high activity of phosphatase during the larval stage is due to the hydrolysis, histogenesis, cell differentiation and transformation. Low activity of the phosphatase in pupae and adults is because of the process of hydrolysis from V instar larval stage to pupae. This is also studied in Lepidopterous.[24] On the basis of the above studies, it is suggested that during metamorphosis midgut tissues undergo gradual degradation under the presence of hydrolases like acid and alkaline phosphatases which are originated from lysosymes in degenerated cells. This is also studied in Anthereae mylilttа, tasar silkworm by Sinha et al. (1991) where ACPH activity was maximum in the 3rd instar and decreased in spinning stage. APH activity was minimum and decrease again in spinning stage.[25]

The changes in ACPH zymograms during development stages revealed a total of 19 bands in KA & PM, and 18 bands in NB_{18}. From the present results, it was clear that ACPH activity was low in KA. ACPH activity was higher in all the races. Furthermore, in the pupal stage the activity was less in male pupae of all the races and it was found to be high in female pupae of all the races. Here also sexual dimorphism has been observed. The present results also agree with the findings of Hedge and Krishnamurthy (1980) where the activity of ACPH was low in eggs but increased gradually towards V instar larvae and it was still decreased in later stages.[26]

The phosphatases are mainly concerned with the digestion of the ingested food and the degradation of the cellular substances.[27] The fact that the silkworm egg shows neither digestive activity nor degradation of cellular substances accounts for low activity of ACPH in the eggs. Further, the larval stage is the only feeding stage in silkworms after which feeding stops. In the pupal and moth stages, there is a lot of resorption of cells and tissues. Hence high phosphatase activity is seen in the larval stage which gradually declines in later stages. The genes which control this mechanism are active in the larval stages and are less active in pupal and get inhibited in the adult stage.

Phosphatases also show specificity. ACPH-5, ACPH-6 and ACPH-7 in KA, ACPH-4, ACPH-5, ACPH-6 and ACPH-7 in case of NB18. ACPH-3, ACPH-5 and ACPH-6 in case of PM, ACPH-4 and ACPH-5 zones in R1 and ACPH-3, ACPH-4 and ACPH-6 zones are non-specific. However, the specificity varies from one race to another race. Here some zones are stage specific also. This specificity/differences in the electrophoretic mobilities of both the enzymes implies divergence in the molecular properties of the protein.[13] Genetic studies made in Drosophila (Beckman and Johnson, show electrophoretic variations in larval APH controlled by a pair of codominent alleles. Subsequently, genetic and developmental relationships between larval and pupal APH were investigatedby Willis and Fox (1968).[28] Genetic inheritance of polymorphic m-APH in Bombyx midgut was studied by Takeda et al., 1992.[13] The functions of both APH and ACPH have been well reported in the silkworm (Sridhar and Bhatt, 1963).[23]

CONCLUSIONS

In conclusion, changes in ACPH zymograms during development stages revealed a total of 19 bands in KA & PM, and 18 bands in NB18. Furthermore, it was evident from the results of present study that ACPH activity was low in KA. ACPH activity was moderate in larval as well pupal stages. Moreover, in the pupal stage the ACPH activity was less in male pupae of all the races and it was found to be high in female pupae of all the races.

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